

Are type IV pili involved in *Vibrio anguillarum* virulence towards sea bass (*Dicentrarchus labrax* L.) larvae?

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ABSTRACT

Vibrio anguillarum, an important bacterial fish pathogen, expresses a variety of virulence factors contributing to its ability to cause vibriosis in fish. Many virulence factors of this pathogen remain however unknown. For example, a type IV pilus system was previously reported to be potentially involved in the virulence of this bacterium but no experimental evidence was reported yet. In this study, complete genome sequencing of *V. anguillarum* strain VIB15, shown to be highly virulent towards sea bass (*Dicentrarchus labrax* L.) larvae, revealed the presence of a *PilA* pilin. A *V. anguillarum* VIB15 *pilA* mutant was constructed and the pathogenicity of this mutant was assessed in a gnotobiotic sea bass system developed for virulence screening. Our results suggest that the *V. anguillarum pilA* gene is not crucial for virulence towards sea bass larvae. Possibly, another type IV pilus system identified in *V. anguillarum*, showing homology to the mannose-sensitive hemagglutinin pilin of *Vibrio cholerae*, may complement the *pilA* mutation. Alternatively, the type IV pilus system has a role in infection of juvenile or adult fish, rather than in the larval phase. As such, further research is required to unravel the potential role of type IV pili in *V. anguillarum* virulence.

Keywords: Virulence; *Vibrio anguillarum*; Fish

Pathogen; Type IV Pili

1. INTRODUCTION

Vibrio anguillarum is an important fish pathogen causing vibriosis in various cultured and wild fish as well as in bivalves and crustaceans. Because of its high morbidity and mortality rate, this hemorrhagic septicemic disease is responsible for severe economic losses in both larviculture and aquaculture worldwide [1-3]. Previous studies have identified several virulence-related genes in *V. anguillarum*, including genes involved in chemotaxis and motility, iron uptake, synthesis of lipopolysaccharides and extracellular products with proteolytic or haemolytic activity (reviewed in [4,5]). Much of the virulence mechanism of *V. anguillarum* remains however poorly understood. In order to get a better understanding of the virulence strategies employed by this pathogen, Rodkum *et al.* [6] identified 40 putative virulence-related genes by random genome sequencing. Focusing on the initial stages of infection, eighteen genes mediating adherence and colonization of bacteria to host cells were identified. Five of these genes were associated with type IV pili, flexible, hair-like filaments on the surface of a wide variety of Gram-negative and Gram-positive genera, making type IV pili the most widespread colonization factors [7]. In addition to the role as adherence factors to living and nonliving surfaces, these pili have an important function in other bacterial processes as well, including flagellum-independent twitching motility, DNA uptake and exchange, biofilm formation, and secretion of extracellular proteins [8,9]. Furthermore, type IV pili

have been identified as important virulence factors in several human, plant and animal pathogens, including several fish pathogens [8,10-14]. Type IV pili possibly contribute to the pathogen's virulence by their roles in adhesion, immune escape, surface motility, microcolony formation, and complement inhibition [8].

The type IV pilus is composed of hundreds of copies of the major pilin subunit and a low abundance of minor pilins required for function and/or assembly of the pilus. All type IV pilins are synthesized as prepilins comprising a leader peptide with an N-terminal consensus motif. Formation of the mature pilin involves cleavage of the leader peptide and methylation of the newly formed N-terminal residue by a specific prepilin peptidase/N-methyltransferase protein. After translocation across the inner membrane, the mature pilin is assembled into a multimeric pilus fiber on a base of minor pilins on the periplasmic side of the inner membrane (**Figure 1**). Minor pilins are pilin-like proteins involved in type II secretion system, which are required for secretion. Finally,

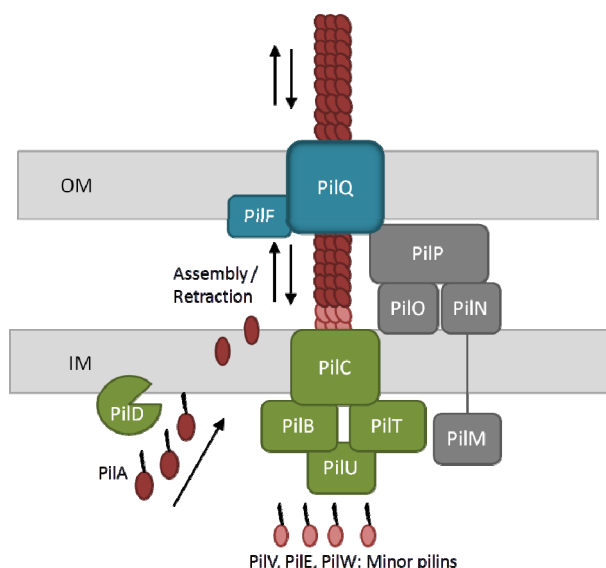


Figure 1. Schematic overview of the type IV PilA pilus system. The major type IV pilin, PilA, is synthesized as a prepilin consisting of a leader peptide with a consensus N-terminal motif. The leader peptide is cleaved and the resulting N-terminal residue is methylated by a specific prepilin peptidase/N-methyltransferase protein, PilD. After translocation across the inner membrane, the mature pilin is assembled into a multimeric pilus fiber on the periplasmic side of the inner membrane. Subsequently, the filament is secreted across the outer membrane through a secretin pore, PilQ. Two different ATPases, PilB and PilT, promote extension and retraction, respectively of the pilus. In addition, a transmembrane protein found in the inner membrane, PilC, and a number of pseudopilins, PilV, PilE and PilW, are involved in the pilin biogenesis. The four sub-complexes shown are the cytoplasmic motor subcomplex (green), the inner membrane alignment subcomplex (grey), the outer membrane secretin pore subcomplex (blue) and the pilus itself (brown) (adapted from [33-35]).

the major pilin is secreted across the outer membrane through a secretin pore.

In the current study, the potential role of the *V. anguillarum* type IV pili in virulence was investigated using a gnotobiotic sea bass (*Dicentrarchus labrax* L.) larvae system.

2. MATERIALS AND METHODS

2.1. Bacterial Strains

Bacteria and plasmids used in this study are listed in **Table 1**. The parental strain for the knockout mutant was *V. anguillarum* VIB15, an O1 serotype strain which was originally isolated from a diseased sea bass in Greece [15]. This strain was made rifampicin resistant (VIB15Rif^R) by natural selection, allowing survival of the bacterium in the gnotobiotic system used in this study. *V. anguillarum* was grown in tryptic soy broth (TSB) (Oxoid, Erembodegem, Belgium) supplemented with 1% NaCl, or in marine broth (MB) (Difco Laboratories, Detroit, USA) for 24 hours at 28°C. *Escherichia coli* strains were grown overnight in Luria Bertani (LB) broth or TSB at 37°C. For solid media, 15 g/l of agar was added. Medium additives were used in the following concentrations when required: 40 mg/ml X-Gal (Invitrogen, Merelbeke, Belgium), 100 µg/ml ampicillin (Ap), 5 µg/ml chloramphenicol (Cm), 10 µg/ml, 100 µg/ml or 200 µg/ml rifampicin (Rif), and 50 µg/ml streptomycin (Sm) (Sigma Aldrich).

2.2. DNA Isolation and Manipulation

Plasmid DNA isolation and PCR purification was performed using QIAprep Spin Miniprep Kit and QIAquick PCR Purification Kit, respectively (Qiagen Inc., Valencia, CA, USA). Genomic DNA was extracted using the phenol/chloroform extraction method as described by Lievens *et al.* [16]. DNA quantity and quality was assessed using the Quant-iTTM PicoGreen[®] dsDNA reagent (Invitrogen).

Standard techniques for restriction endonuclease digestion, ligation, gel electrophoresis, and southern hybridization were performed as described by Sambrook and Russell [17]. Restriction and modification enzymes were obtained from Bioké (Leiden, The Netherlands) and Roche Diagnostics (Vilvoorde, Belgium). DNA quantity and quality was assessed using the Nanodrop spectrophotometer or the Quant-iTTM PicoGreen[®] dsDNA reagent (Invitrogen).

2.3. PCR

PCR amplification was performed in a total volume of 20 µl containing 0.5 µM of each primer, 0.15 mM of each deoxynucleoside triphosphate (Invitrogen), 2.0 U

Table 1. Bacterial strains, plasmids and primers used in this study.

Strains, Plasmids, Primers	Relevant characteristics	Reference
Bacterial strains		
<i>Escherichia coli</i>		
TOP10	F- <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>lacZ</i> Δ M15 Δ <i>lacX</i> 74 <i>recA1</i> <i>araD</i> 139 Δ (<i>ara-leu</i>)7697 <i>galU galK rpsL</i> (StrR) <i>endA1 nupG</i>	Invitrogen
S17-1 λ -pir	<i>thi pro hsdR-hsdM+</i> <i>recA</i> RP4 2-Tc::Mu-Km::Tn7(TpR SmR) λ -pir lysogen, Strain for biparental conjugation	[51]
<i>Vibrio anguillarum</i>		
VIB15	Wild type isolate from sea bass, Greece, Serotype O1	[15]
VIB15 ^{Rif^R}	Rifampicin resistant VIB15	This study
VIB15 ^{Rif^R} Δ <i>pilA</i>	Rifampicin resistant VIB15 with deletion within <i>pilA</i>	This study
Plasmids		
pCR [®] 2.1	TA-cloning vector	Invitrogen
pDS132	Suicide plasmid for <i>V. anguillarum</i> , mobilizable from <i>E. coli</i> S17-1; containing <i>sacB</i> for counterselection with sucrose, CmR	[52]
Primers		
van-ami8	5'-ACATCATCCATTGTAC-3'	[43]
van-ami417	5'-CCTTATCACTATCCAAATTG-3'	[43]
FPDS132	5'-CACTTGCCGTCCATATACAC-3'	This study
FPO_PiA ^a	5'-TACGCATGCTTAGAAGAGGCGATTCAAGC-3'	This study
RP_PiA ^b	5'-TCGCAGGTAGGTTGGTTAGCATTTGTATTCTTAGCTACAGGTAAAACCACTTTGTTTCG-3'	This study
FP_PiA ^b	5'-ATGAACACAAATAAGAAAACGAAACAAAGTGGTTTACCTGTAGCTAAGAAATACAAATGC-3'	This study
RPO_PiA ^a	5'-ACCGAGCTCAGTTCGCCAGTTCATCTTGG-3'	This study
PRTPiID	5'-AACGGTGCTTTTTCCGCTG-3'	This study
PiAF	5'-TCCATCCTTATGCGTTCAAAC-3'	This study
PiAR	5'-TGTCTGGGTAACACCTGTG-3'	This study
PiBF	5'-ATCACCCGTTACCAAGCAC-3'	This study
PiBR	5'-GTGCTGCTTTTACGGCAATTCG-3'	This study
PiCF	5'-AAAGAACAGCACATTCAGATC-3'	This study
PiCR	5'-GCTTCGGTATTACGCATCG-3'	This study
PiDF	5'-CTTTCCCGAATATCACATTGC-3'	This study
PiDR	5'-ACAGATGACATGAGAATGATG-3'	This study

^aThe nucleotides underlined represent recognition sites for restriction enzyme *SacI* (GAGCTC) or *SphI* (GCATGC); ^bItalic sequences represent overlapping sequences to create a fusion of the *pilA* upstream and downstream region.

Taq DNA polymerase (Bioké), 10× ThermoPol Reaction Buffer (Bioké), and 1 μ l of DNA solution (1 ng/ μ l, as measured by a Nanodrop spectrophotometer). All amplifications were performed using a Bio-Rad T100 thermal cycler with initial denaturation at 94°C for 5 min, followed by 30 cycles of 30 sec at 94°C, 30 sec at 59°C, and 30 sec at 72°C, with a final extension step at 72°C for 10 min. In case Platinum[®] Pfx DNA Polymerase (Invitrogen) was used, elongation temperature was changed to 68°C.

2.4. Genome Sequencing

V. anguillarum strain VIB15 was grown overnight on marine agar (MA) (Difco) at 28°C. Following DNA iso-

lation, a paired-end library (2 × 100 bp, 500 bp inserts) was prepared for sequencing on the Illumina HiSeq 2000 platform (Illumina, San Diego, CA, USA) at Genomics Core (UZ Leuven, Belgium) according to the manufacturer's instructions. Sequencing reads were subjected to quality filtering using NGS-QC Toolkit [18]. Low quality reads (reads with 70% of the nucleotides having a Phred quality score below 30) were removed, and high quality reads were trimmed to remove the first and the last 13 nucleotides. Trimmed reads were *de novo* assembled using SOAPdenovo v1.05

(<http://www.soap.genomics.org.cn/soapdenovo.html>).

Assembled contigs were subsequently scaffolded by SSPACE [19], and additional gaps were closed by Gap-

Filler [20]. The assembled genome sequence was annotated by the RAST server [21]. Homology searches were performed by using the BLAST program [22].

2.5. Bacterial Mating

Plasmids were introduced into *V. anguillarum* VIB15Rif^R by conjugation procedures described previously [23]. Briefly, overnight cultures of *V. anguillarum* and *E. coli* S17-1 λ -pir were mixed at a ratio of 10:1 in 2.5 ml 10 mM MgSO₄. The cell mixture was vacuum filtered onto a 0.22- μ m-pore-size filter, which was placed on tryptic soy agar (TSA) and allowed to incubate overnight at 25°C. Following incubation, the cells were harvested and plated on LB agar plates containing 200 μ g/ml Rif and 5 μ g/ml Cm and allowed to incubate at 25°C until *V. anguillarum* colonies were observed.

2.6. Construction of Chromosomal Mutation

To generate a *pilA* in-frame deletion fragment of strain VIB15Rif^R, an asymmetric overlap extension PCR (AOE-PCR) was performed (Figure 3). The upstream and downstream regions of the *pilA* gene were amplified asymmetrically using primers FPO_PilA/RP_PilA and FP_PilA/RPO_PilA, respectively (Table 1), at a 1:10 inner:outer primer ratio and Platinum[®] Pfx DNA Polymerase (Invitrogen) according to the manufacturer's instructions. The inner primer for amplification of the region upstream of *pilA*, RP_PilA, includes 40 nucleotides at the 5' end, which is complementary to the downstream region and vice versa for primer FP_PilA, generating an excess of single-stranded DNA with overlapping 3' ends. Both fragments hybridized and functioned as a template for PCR amplification of the desired in-frame deletion fragment. For this PCR reaction 1 μ l of each reaction, Taq DNA polymerase and the outer primers FPO_PilA and RPO_PilA were used. The resulting PCR product was directly cloned into the pCR[®]2.1 cloning vector using the TA cloning kit (Invitrogen). Following *SphI*/*SacI* digestion of the resulting plasmid, gel purification and ethanol precipitation, the *pilA* knock-out fragment was ligated into the *SphI*/*SacI* site of the dephosphorylated pDS132 suicide vector. The ligation reaction was subsequently purified using the QIAquick PCR Purification Kit (Qiagen Inc.), electroporated into *E. coli* S17-1 λ -pir and introduced into VIB15Rif^R by bacterial mating. VIB15Rif^R single recombinants were selected on LB plates containing chloramphenicol and incorporation of the plasmid was confirmed with PCR (FPDS132/RPO_PilA). In the next step, double homologous recombinants were selected on LB5 (LB with 0.5% NaCl) containing 100 μ g/ml Rif and 10% sucrose for *sacB* counter selection. Gene replacement in these strains was confirmed by PCR using *pilA*-specific primers PilAF and PilAR and south-

ern hybridization.

2.7. RT-PCR

Total RNA was extracted from *V. anguillarum* VIB15Rif^R and the *pilA* knockout mutant after overnight growth on MA using the Spectrum[™] Plant Total RNA kit (Sigma Aldrich). Subsequently, cDNA was synthesized using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer's instructions, and the *pil* locus-specific reverse primer PRTpILD. The obtained cDNA was analyzed by PCR for the presence of transcripts from *pilA*, *pilB*, *pilC* and *pilD*. The primers used for amplification are listed in Table 1.

2.8. Virulence Testing

Both VIB15Rif^R strain and *pilA* knockout mutant were subjected to a virulence assay in a standardized gnotobiotic model system with axenic European sea bass (*D. labrax* L.) larvae as hosts, as described previously [24, 25]. Briefly, eggs of *D. labrax* were obtained from natural spawning at the hatchery of Ecloserie Marine de Gravelines (France) followed by a standard disinfection protocol generating germ-free eggs. After hatching, sea bass larvae were challenged with a suspension of 10⁵ cfu/ml *V. anguillarum*. Prior to inoculation, these bacterial strains were grown in 10% MB with addition of NaCl and 10 μ g/ml Rif, and incubated on a horizontal shaker at 120 rpm at 16°C \pm 0.5°C for 2 days. For each challenge, 10 replicates were prepared and the vials were rotated at 4 rpm tangential to the axis of the vials. On DAH (days after hatching) 6, 8, 10 and 12 (*i.e.* 3 - 9 days post-exposure), survival of the sea bass larvae was monitored by microscopic analysis. Non-inoculated germ-free larvae that were kept under similar conditions served as a control. Throughout the experiment, eggs and larvae were kept at a salinity of 36 g/l in a temperature controlled room at 16°C \pm 0.5°C in constant dim light (10 candela steradian/m²). The experiment was approved by the ethical committee of Ghent University (no. EC2012/071). Two independent repeats of this gnotobiotic sea bass challenge experiment were performed. Statistical analysis of the larval survival data was performed by means of R v2.12.1. Survival was reported as mean values (% survival) \pm standard error of the mean (SEM). Data were tested for normality and subjected to non-parametric tests. Wilcoxon rank-sum test was used to compare the survival of the sea bass larvae. Significance was accepted at $p < 0.05$.

3. RESULTS

3.1. Identification of *V. anguillarum* Type IV Pilus Biogenesis Gene Cluster

To identify genes involved in the biogenesis of the *V.*

anguillarum type IV pilus system, the complete genome sequence of *V. anguillarum* strain VIB15 was determined. In a previous study using a standardized gnotobiotic system, it was demonstrated that strain VIB15 is highly virulent towards sea bass (*D. labrax* L.) larvae [25].

The genome sequence revealed that the gene encoding the major pilin, *pilA*, comprises 427 bp and is arranged in a typical type IV pilus biogenesis cluster, *pilABCD*. *pilB* encodes a cytoplasmic protein required for ATP binding and hydrolysis, *pilC* an inner membrane protein, and *pilD* the prepilin peptidase/N-methyltransferase protein. Downstream of this cluster, a fifth open reading frame, *yacE*, has been identified, encoding a dephospho-CoA kinase. Although the same gene organization is found in many other bacteria, the role of *yacE* in the type IV pilus assembly is unknown [26,27]. Upstream of the *V. anguillarum pilABCD* cluster, the *nadC* gene, encoding a quinolinate phosphoribosyltransferase, was identified. The *nadC-pilABCD-yacE* gene organization was also observed in *V. cholerae* and *V. vulnificus*, indicating a conserved gene synteny [26,28]. The protein sequences of PilA to PilD are 99% - 100% identical to the corresponding proteins in *V. anguillarum* strain 775, and shows high amino acid identity to the *V. vulnificus* homologs (42%, 75%, 75%, and 71%, respectively) [28-30].

Four additional clusters required for pilus biogenesis and assembly, *pilMNOPQ*, *pilVWE*, *pilUT*, and *pilF*, were identified on chromosome I (Figure 2). The *pilVWE* cluster encodes minor pilins which form the base for the major pilin, PilA. Recently, Giltner *et al.* [31] reported that these minor pilins are also incorporated into the growing pilus fiber, supporting an additional role of these minor pilins in optimal pilus assembly. The *pilQ* gene encodes a secretin through which the major pilin is secreted across the outer membrane. Assembly of this secretin requires its cognate pilotin, PilF, an outer membrane lipoprotein involved in the correct localization and multimerization of PilQ [32]. Furthermore, in contrast to PilB, which is involved in the assembly and extension of type IV pilin, PilT and PilU promote disassembly and retraction of the pilin. Finally, PilM, PilN, PilO and PilP form the bridge between the cytoplasmic and outer membrane components of the type IV pilus system [33, 34]. Figure 2 illustrates that the different genes and gene clusters involved in type IV pilus biogenesis, are scattered throughout chromosome I of *V. anguillarum*, which is also the case in other organisms [7,14,33].

Focusing on the major pilin, PilA, considerable homology to the type IV pilin precursor proteins of many other bacteria was observed [11,26,28,35,36]. Typically, the N-terminal region of the premature PilA of *V. anguillarum* VIB15 is dominated by a short, hydrophilic leader peptide and shows the presence of the conserved

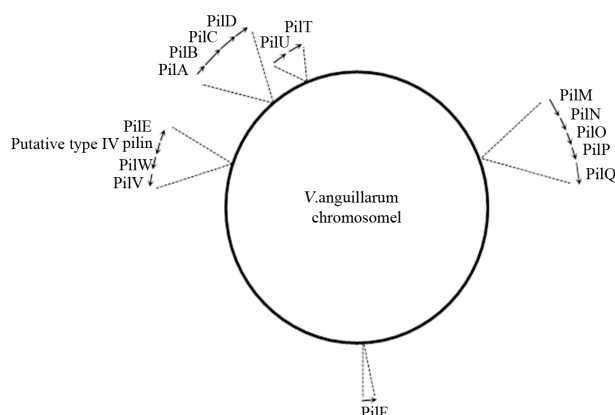


Figure 2. Schematic representation of chromosome I of *V. anguillarum* VIB15 mapping the genes involved in PilA type IV pilus system. The arrows indicate the approximate size and orientations of the genes.

consensus cleavage site with the GFTLIE amino acid motif, which is identical in PilA of several other *Vibrio* species such as *V. cholerae* [26]. After cleavage of the leader peptide, the mature PilA contains a very hydrophobic N-terminal segment of about 20 - 25 residues. The C-terminal domain of PilA is less conserved, although it contains a characteristic pair of cysteines, responsible for a disulfide-bonded loop containing a receptor-binding site. Involvement of this binding-site either in intersubunit contacts throughout the length of the pilus fiber or host-cell adherence have been proposed [37-40].

3.2. Construction of a Non-Polar *pilA* Mutant Strain

To examine the potential role of PilA in *V. anguillarum* infection, a non-polar knockout mutant of strain VIB15Rif^R was generated. Using AOE-PCR [41], an in-frame deletion of 378 bp of the *pilA* gene was constructed (Figure 3). First, it was verified that the growth in MB was not affected in the mutant strain (data not shown). Second, RT-PCR was used to verify that the pilus biogenesis genes downstream of *pilA*, i.e. *pilB*, *pilC* and *pilD*, are still expressed in the knockout strain. The cDNA generated by a reverse primer downstream of *pilD* (PRTPilD) was examined for the presence of *pilA*, *pilB*, *pilC*, and *pilD* transcripts using specific primers internal to each gene (Table 1). As can be seen in Figure 4, the presence of PCR fragments of the expected size for strain VIB15Rif^R, corresponding to transcription of *pilA* (364 bp), *pilB* (979 bp), *pilC* (882 bp), and *pilD* (567 bp), was confirmed. However, the *pilA* mutant strain was clearly deficient in *pilA* transcription, confirming that the deletion in the *pilA* gene resulted in the loss of transcription of the target gene but did not affect transcription of the downstream genes.

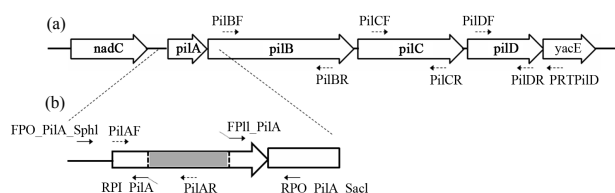


Figure 3. Gene organization of the type IV PilA pilus biogenesis gene cluster of *V. anguillarum* VIB15 (a); The primers used for fusing the *pilA* upstream and downstream regions are indicated solid arrows, while primers used for confirmation of the *pilA* mutation are indicated with dashed arrows (b).

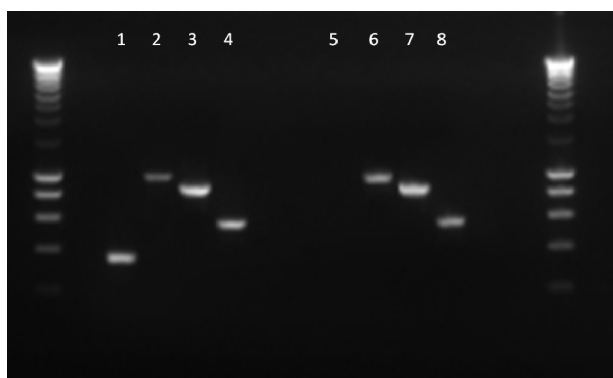


Figure 4. Detection of *pilA*, *pilB*, *pilC* and *pilD* transcripts by RT-PCR in parental *V. anguillarum* strain VIB15Rif^R (lanes 1 to 4, respectively) and *pilA* knockout strain (lanes 5 to 8) using specific internal primers for each gene. Smartladder (Eurogentec) was used as molecular weight marker.

3.3. Assessment of the Virulence of the *pilA* Mutant

To investigate the role of the PilA pilus in the virulence of *V. anguillarum*, both wild-type strain and the *pilA* knockout mutant were subjected to virulence assessment in the gnotobiotic sea bass larvae system. Previous studies have demonstrated the usefulness of this experimental system to assess the virulence of bacterial pathogens *in vivo* [24,25,42]. After challenging the sea bass larvae with *V. anguillarum*, larval survival was monitored at DAH 6, 8, 10 and 12. The experiment was terminated at DAH 12 as the sea bass larvae died of starvation in prolonged experiments. The averaged results of both challenging experiments are presented in Figure 5. Survival on DAH 12 of the sea bass larvae challenged with the parental strain or the PilA knockout mutant ($16\% \pm 4\%$ survival and $10\% \pm 3\%$ survival, respectively) was significantly different from the control group ($72\% \pm 5\%$). However, on DAH 12, no significant difference in survival could be observed between the wild-type VIB15 strain and the *pilA* knockout mutant. The same could be observed on DAH 6, DAH 8 and DAH 10. These results suggest that the PilA type IV pilus is not a crucial virulence factor in *V. anguillarum* VIB15. To confirm that mortality was caused by the tested *V.*

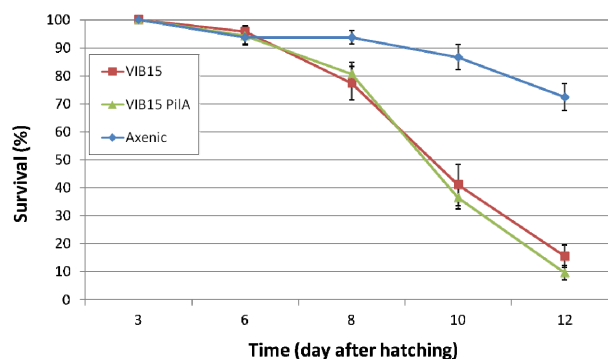


Figure 5. Survival (in percentage) of sea bass larvae during the gnotobiotic experiment after challenging the larvae with the wild-type *V. anguillarum* strain VIB15Rif^R and the *pilA* knockout strain. The averaged results of two independent repeats are presented. Error bars represent mean \pm SEM ($n = 20$).

anguillarum strains and not by contamination, the identity of the inoculated strains was confirmed by isolating the bacteria again at the end of the experiment (DAH 12) followed by PCR using the *V. anguillarum*-specific primers van-ami8/van-ami417 and the *pilA*-specific primers PilAF/PilAR [43].

4. DISCUSSION

The PilA type IV pilus system has been previously identified as an important virulence factor in several fish pathogens. It has for example been reported that the *Aeromonas salmonicida* PilA homologue, TapA, was required for virulence in rainbow trout (*Oncorhynchus mykiss* (Walbaum)) and Atlantic salmon (*Salmo salar* L.) [11,14]. In addition, the *pilA*-encoded type IV pilus of *V. vulnificus* contributed significantly to the persistence in *Crassostrea virginica* oysters [13]. In several other pathogenic bacteria, the type IV pilus has been shown to be crucial for virulence [7,8,12,44]. Therefore, the putative role of this type IV pilus system in the virulence of *V. anguillarum* was investigated in the current study.

The complete genome sequence of *V. anguillarum* strain VIB15, previously reported to be highly virulent towards sea bass larvae, revealed the presence of genes and gene clusters involved in secretion and assembly of a type IV pilus system (Figure 2) [7,35]. PilA, the major pilin, is encoded by the *pilABCD* gene cluster, showing high similarity to the *pilABCD* gene cluster of *V. vulnificus* [28], and *V. cholerae* [26], and the *tapABCD* gene cluster of *A. salmonicida* [11]. To investigate the potential role of this type IV pilus system in the virulence of *V. anguillarum*, a non-polar knockout mutant of the major pilin, PilA, of strain VIB15 was successfully created. The virulence of the *pilA* mutant was assessed using a standardized gnotobiotic system with axenic European sea bass larvae as host organism [24]. This standardized gnotobiotic system has proven its strength as a model to

evaluate the virulence of *V. anguillarum* *in vivo* [24,25, 42]. Unexpectedly, the virulence assay revealed no significant difference in larval survival between the wild-type strain and the *pilA* knockout mutant. A possible explanation for this observation is the identification of a second type IV pilus system in the *V. anguillarum* genome, the mannose-sensitive hemagglutinin pilus (MSHA) system, which may complement the PilA deficiency. This system is very similar to the *V. cholerae* MSHA pilus and has been previously identified in other *Vibrio* species such as *V. vulnificus* and *V. parahaemolyticus* [45]. However, in *V. vulnificus*, the presence of the MSHA pilus was not able to complement for the loss of the PilA pilus, as the *pilA* knock out mutant was severely attenuated in biofilm formation and virulence in mice [28]. Moreover, genes responsible for the biosynthesis of this MSHA pilus were repressed during *V. cholerae* infection in infant mice [46].

Alternatively, the PilA pilus might have a role in infection of juvenile or adult fish, rather than the fish larvae. In this regard, it has been reported that the expression of the *pilA* gene of *V. cholerae* and *V. parahaemolyticus* is induced by chitin, a polymer found in high abundance in fish scales and oyster shells [13,47,48]. This may indicate that in the gnotobiotic larvae system used in this study, the *V. anguillarum pilA* expression is lower, suggesting that the PilA pilus doesn't play a crucial role in virulence towards sea bass larvae. The influence of type IV pilin on the virulence of *A. salmonicida* in rainbow trout and Atlantic salmon was examined using juveniles covered with scales instead of larvae without scales [11,14,49]. In addition, the presence of chitin in the shell of the oysters might explain the role of PilA of *V. vulnificus* in oysters [13]. Finally it is possible that the type IV pilin of *V. anguillarum* strain VIB15, PilA, is not involved in the virulence of the pathogen but in the persistence of the bacterium in the aquatic environment. Chiavelli *et al.* [50] for instance, reported that the type IV pilus of *V. cholerae* promotes attachment to zooplankton exoskeletons as a survival strategy in the aquatic environment.

In conclusion, although type IV pili have been identified as important virulence factors in several human, plant and animal pathogens, including several fish pathogens, the data presented here suggested that PilA had no crucial role in the virulence of *V. anguillarum* VIB15 towards sea bass (*D. labrax* L.) larvae. Some possible explanations have been proposed but further studies are necessary to elucidate the role of both type IV pilus systems in fish and the environment.

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